Rec'd PCT/PTO 20 AUG 2001
PCT/GB00/00582
09/913853

PLURIPOTENTIAL CELLS-1

The invention herein described relates to isolated pluripotential cells, comprising at least part of the cytoplasm from a teratocarcinoma cell and a nucleus of a somatic cell; methods to prepare such cells; therapeutic compositions of said cells; and uses thereof.

Animal embryonic development is a highly regulated development process that combines cell proliferation and cell/tissue differentiation to produce an intact organism. The co-ordination of cell proliferation and differentiation is, and has been, the subject of intense research and the information derived from this has contributed to our understanding of cell function and disease. For example and not by way of limitation, regulation of gene expression, cell differentiation, oncology, teratology.

Mammalian embryonic development is remarkably conserved during the early stages. Post fertilisation the early embryo completes four rounds of cleavage to form a morula of 16 cells. These cells complete several more rounds of division and develope into a blastocyst in which the cells can be divided into two distinct regions; the inner cell mass, which will form the embryo, and the trophectoderm, which will form extra embryonic tissue, (eg placenta).

Those cells that form part of the embryo up until the formation of the blastocyst are said to be totipotent (e.g. each cell has the developmental potential to form a complete embryo and all the cells required to support the growth and development of said embryo).

During the formation of the blastocyst. the cells that comprise the inner cell mass (ICM) are said to be pluripotential (ie each cell has the developmental potential to form a variety of tissues).

Embryonic stem cells may be principally derived from two embryonic sources. Pluripotential cells isolated from the inner cell mass are termed embryonic stem cells (ES cells). An alternate source of pluripotential cells is derived from primordial germ cells isolated from the mesenteries or genital ridges of days 8.5-12.5 *post coitum* embryos which would ultimately differentiate into germ cells. These pluripotential cells are referred to as embryonic germ cells (EG cells). Each of these types of pluripotential cell has the similar developmental potential with respect to differentiation into alternate cell types.

It is important to note that an intact embryo cannot be produced from a single pluripotential cell (eg either an ES or EG cell). Therefore a pluripotential cell has an increased commitment to terminal differentiation when compared to a totipotent cell.

Until very recently *in vitro* culture of human ES cells was not possible. The first indication that conditions may be determined which could allow the establishment of human ES cells in culture is described in WO 96/22362. The application describes cell lines and growth conditions which allow the continuous proliferation of primate ES cells which exhibit a range of characteristics or markers which are associated with stem cells having pluripotent characteristics.

For example, and not by way of limitation, the expression of specific cell surface markers SSEA-3 (+), SSEA-4 (+), TRA-1-60 (+), TRA-1-81 (+) (Shevinsky *et al* 1982; Kannagi *et al* 1983; Andrews *et al* 1984a; Thomson *et*

al 1995) and alkaline phosphatase (+). In addition the established primate cell lines disclosed in WO 96/22362 have stable karyotypes and continue to proliferate in an undifferentiated state in continuous culture. The primate ES cell lines also retain the ability, throughout their continuous culture, to form tissues derived from all three embryonic germ layers (endoderm, mesoderm and ectoderm).

More recently Thomson *et al* (Science 282: 1145-1147, 1998) have published conditions in which human ES cells can be established in culture. The above characteristics shown by primate ES cells are also shown by the human ES cell lines. In addition the human cell lines show high levels of telomerase activity, a characteristic of cells which show the ability to divide continuously in culture.

An alternative source of pluripotential embryonic stem cells are those derived from primordial embryonal germ cells (EG cells) which are located in the mesenteries or genital ridges of embryos. WO 98/43679 describes the isolation of EG cells from the gonadal or genital ridges of human embryos. EG cells described in WO 98/43679 exhibit features in common with primate and human ES cells, (eg expression of cell surface markers, continuous proliferation in culture in an undifferentiated state, normal karyotype and the ability to differentiated into selected tissues under defined conditions).

It is evident that the use of *in vitro* cultures of pluripotential stem cells, especially human cells, has important ramifications for both basic research (eg as a model for studying gene expression and/or tissue differentiation) and in transplantation and/or replacement therapies for tissues which have been damaged either through injury or disease. The establishment of *in vitro* cultures of human ES and EG cells is a major step toward realising the full

potential of this technology; because of their pluripotent nature ES and EG cells may be capable of differentiating under controlled conditions into a variety of cell types and/or tissues and organs that could have a wide variety of applications. For example, and not by way of limitation, replacement of damaged and/or diseased coronary and/or major arteries; replacement of damaged and/or diseased organs (eg as a result of kidney disease, (eg cirrohosis), diabetes, various autoimmune diseases); replacement of damaged neurones (eg Alzhiemers disease, Parkinsons disease, spinal injuries) or cancer. It will also be apparent to one skilled in the art that diseases such as AIDS may benefit from from tissues derived from ES or EG cells. The depletion of T-cells through virus induced cell death is the major contributory factor to the immuno-compromised state of AIDS suffers.

However, there are practical and ethical difficulties associated with the use of material derived from human embryos. Morever, such allogeneic material, if transplanted into another human, may illicit a severe immune reaction in the host and be thus destroyed.

It has been known for many years that amphibian somatic cell nuclei retain their ability to give rise to entire organisms when they are transplanted into egg cells which have had their nucleus removed or inactivated (Gurdon 1974). Thus determination of the pluripotent of these cells must be controlled by the egg cytoplasm which was able to in effect reprogramme the somatic cell nucleus into a totipotent state.

Mammalian somatic cell nuclei have also been shown to retain this placicity and can be reprogrammed when transferred to enucleated oocytes, (Campbell *et al*; Wakayama *et al)*

Moreover nucleated mouse ES cells have been shown to be able to reprogramme somatic cell nuclei, although in this case, a heterokaryon was produced containing the cytoplasm and nuclei from both types of cells so it is difficult to determine the actual mechanism of action of the reprogramming state.

In all these examples, althought the material produced is genetically identical to the somatic cell donor, these somatic cells were reprogrammed by cellular elements are derived from either ooctyes or ES cells and again, in human this poses practical and ethical concerns.

Embryonal carcinoma cells derived from teratomas are also able to reprogramme somatic cell nuclei in order to produce cells with pluroptential characteristics.

Teratomas, tumours that contain a wide range of more or less organised tissues have been known in humans for many hundreds of years. They typically occur as gonadal tumours of both men and women, but they also occur in other sites. The gonadal forms of these tumours are generally believed to originate from germ cells, and the extra-gonadal forms, which typically have the same range of histology, are widely thought to arise from 'mis-placed' germ cells that have migrated incorrectly during embryogenesis. However, a non-germ cell origin from persisting embryonic stem cells may be considered in some cases, especially for teratomas occurring in the new born.

Because of the presumption of a germ cell origin in most cases, teratomas are generally classed as germ cell tumours (GCT), which also manifest a range of other histological types, including seminoma (often called dysgerminoma in females), embryonal carcinoma (EC), yolk sac carcinoma and

choriocarcinoma. GCT may contain any combination of these tissue types, with or without elements of teratoma. Combinations of teratoma and EC are frequently described as teratocarcinoma. Commonly, human GCT are divided into pure seminomas, in which none of the other histological types occur, and non-seminomatous GCT (NSGCT), which may contain any combination of histological types, with or without elements of teratoma. Confusingly, NSGCT may also contain elements of seminoma.

Ovarian GCT are most commonly benign and contain only well differentiated somatic tissues that may include bone, muscle, nerve. Often well-organised tissues are found, including teeth and hair. By contrast, human testicular GCT are always malignant containing any combination of the tumour tissue types discussed above. Any teratoma elements present may be less well organised than in benign human ovarian teratomas, but somatic cell types such as nerve, muscle, bone and cartilage may be quite recognisable. Testicular GCT are rare but have a peak incidence after puberty, being the most common form of cancer in young men between the ages of 20 - 35.

Early histopathological studies of human GCT led to the proposal that EC cells resemble early embryonic cells and are the stem cells that give rise to all the other cell types in GCT, with the exception of seminoma. (In the currently prevailing view, seminoma more closely resembles the primordial germ cells from which these tumours arise, and so may represent an earlier stage in tumour development and progression). Detailed experimental study of GCT became possible with the discovery that male laboratory mice of the strain 129 develop spontaneous testicular teratocarcinomas. Studies of these mice confirmed that these tumours had a germ cell tumour origin, and indeed arose from primordial germ cells (PGC) at about the time that they migrated into the genital ridge of the developing embryo - 11 - 13 days of gestation in the

laboratory mouse. Evidence was also obtained supporting the hypothesis that EC cells are pluripotent stem cells that are able to generate the whole range of differentiated cells found in teratocarcinomas. Subsequently it was found that similar teratocarcinomas and teratomas could be derived from embryos that have been transplanted to ectopic sites.

For reasons that remain unclear, seminoma has not been observed in laboratory mice, and so no animal model of this tumour exists. Spermatocytic seminoma do occur in dogs but, although these also occur in old men, they appear to be a quite different tumour type with different aetiology to the GCT.

EC cell lines that can be maintained in vitro have been derived from several mouse teratocarcinomas. Some of these EC cell lines were found to retain a pluripotent phenotype and could be induced to differentiate into a range of cell types, either by transplantation back to a syngeneic mouse, in which case a teratocarcinoma would be formed, or by various manipulations in vitro. Some EC cell lines differentiated spontaneously when allowed to grow to confluence. Others required a feeder layer of irradiated or mitomycin-treated cells if they were to retain an EC phenotype, and they differentiated spontaneously if removed from the feeder cells. In a number of cases, it was found that maintaining EC cells in suspension culture for several days forced them to form floating clumps of cells that became vesiculated and began to differentiate. These floating clumps were known as embryoid bodies; a wide range of differentiated cell types, including nerve and muscle, would grow out from these if they were subsequently allowed to attach to a tissue culture surface. It was also found that a number of chemical agents, most strikingly retinoic acid, also induced the differentiation of many murine EC cells into a range of cell types. The precise cell types formed in response to any of these treatments depended upon the particular EC cell line.

Access to EC cell lines allowed detailed characterisation of their properties and pattern of marker expression. Several surface antigens, notably the 'F9 antigen' and Stage Specific Embryonic Antigen-1 (SSEA-1) were identified as characteristically expressed by mouse EC cells. It was further noted that these cells did not express class 1 major histocompatibility (MHC) antigens - H-2, in the mouse. These features, as well as morphology and their capacity to differentiate suggested that murine EC cells resembled cells of the inner cell mass or primitive ectoderm of the early mouse embryo. This resemblance was confirmed by the finding that some EC cells could differentiate and participate in normal embryonic development, when transferred to a blastocyst of an early mouse embryo, which was then re-implanted in a pseudo-pregnant mother and allowed to develop to term. In some cases the chimeras developing from such EC cell ↔ embryo combinations were normal with extensive contributions from the EC cell component; in some cases the chimeras subsequently developed teratocarcinomas, indicating that the tumour phenotype of the EC cells had not been fully suppressed. Only in a very small number of cases was germ cell chimerism reported.

EC cells have been shown to have many of the features which characterise ES and/or EG cells. EC cells are relatively easy to establish in culture, express cell surface markers associated with ES and/or EG cells, can be maintained in continuous culture in an undifferentiated state and have the potential to differentiate into selected tissues both *in vivo* and *in vitro*. However what is also evident is that EC cells contain a mutation, or mutations, in genes (oncogenes) which result in the additional undesirable feature that the cells retain the potential to form tumours.

It has been known for several years that selected chemical treatments of cells in culture can result in cells extruding nuclei resulting in the formation of

separate nuclear and cytoplasmic parts termed karyoplasts and cytoplasts, respectfully. It is well known in the art that the separated parts of the cell may be reconstituted via cell fusion. For example, and not by way of limitation it is possible to produce a cytoplast from one cell and fuse the cytoplast to a selected cell to form a cytoplasmic hybrid, or as is commonly known, a cybrid. In addition it is also possible to fuse the karyoplast to a selected cell to form a nuclear hybrid. The nuclei fuse after nuclear membrane breakdown during mitosis and reconstitute after cytokinesis to form a polyploid or anueploid nucleus. These techniques are well known in the art and will not be detailed extensively at this stage.

We have prepared cytoplasts derived from EC cells and fused the cytoplasts to form cybrids with selected somatic cells. The aim of this approach is to re-programme the differentiated somatic cell nucleus, through contact with factors located in the EC cytoplasm, so that, the cybrid de-differentiates and so takes on the characteristic features of a pluripotential cell. This then provides the basis for the establishment of pluripotential cell lines which, upon exposure to various differentiation factors, can lead to the production of selected differentiated tissue for use in, amongst other things, transplantation therapy.

Advantageously there is no requirement to use harvested embryonic cells to derive the cytoplasts. Therefore any ethical issues with respect to the use of embryos in this way is circumvented. In addition the establishment of EC cells in culture is a relatively amenable task when compared to the problems of establishing human stem cell cultures *in vitro*. Finally the removal of the EC cell nucleus from the donating EC cell results in no transfer of genetic material carrying potential oncogenes to the cybrid cell so formed.

It is therefore an object of the invention to provide a pluripotential cell that is not derived from embryonic tissue from a primary source.

It is a further object of the invention to provide methods of combining at least part of the cytoplasm of an EC cell with a somatic nucleus.

It is yet a further object of the invention to provide a pluripotential cell having the capacity to differentiate into selected tissues upon exposure to appropriate factors.

According to a first aspect of the invention there is provided a cell comprising at least part of the cytoplasm derived from at least one embryonal carcinoma cell combined with at least the nucleus of at least one somatic cell.

In a preferred embodiment of the invention said cell, ideally a cybrid, is characterised by the possession of at least one pluripotential characteristic.

We believe that the acquisition of this pluripotential characteristic is as a result of the re-programming of said somatic nucleus.

It will be apparent to those skilled in the art that the cell of the invention may be derived, most preferably, by the creation of a cybrid; but an alternative option involves the fusion of a somatic cell with an EC cell. Clearly this latter option is not preferred because of the potentially oncogenic genome (gene) of the donating EC cell. Preferably the further step of removing the EC nucleus is described hereinafter.

Ideally said pluripotential characteristic includes the ability to differentiate into at least one selected tissue type, preferably upon exposure to at least one differentiation factor.

Alternatively, or additionally, said pluripotential characteristic includes the ability of said cell to proliferate in culture in an undifferentiated state.

In yet a further preferred embodiment of the invention said cell has the capacity to proliferate in continous culture in an undifferentiated state for at least 6 months and ideally 12 months.

Alternatively or additionally said pluripotential characteristic includes the expression of at least one selected marker of pluripotential cells.

It is well known in the art that pluripotential cells express a number of genes not typically expressed by differentiated cells. These are valuable tools to monitor whether the EC cytoplasm has re-programmed a somatic cell nucleus. One such example is Oct4.

In a preferred embodiment of the invention said selected marker is expression of the Oct4 gene.

In yet still a further preferred embodiment of the invention said selected marker is a cell surface marker. Preferably said cell surface marker is selected from the group including SSEA-1 (-); SSEA-3 (+); SSEA-4 (+); TRA-1-60 (+); TRA-1-81 (+); alkaline phosphatase (+).

Alternatively, or additionally, said pluripotential characteristic includes the presence of telomerase activity in said pluripotential cell. Ideally said telomerase activity is correlated with extension of telomeres.

For the sake of clarity, telomerase enzymes add, *de novo*, repetitive DNA sequences to the ends of chromosomes. These ends are referred to as

telomeres. For example the telomeres of human chromosomes contain the sequence '5 TTAGGG 3' repeated approximately 1000 times at their ends. In young, dividing cells the telomeres are relatively long. In aging, or non-dividing cells, the telomeres become shortened and there is a strong correlation between telomere shortening and capacity to proliferate. Methods to increase the length of telomeres to increase proliferative capacity are known in the art and are described in WO9513383.

Alternatively, or additionally, said pluripotential characteristic includes the presence of a chromosomal methylation pattern characteristic of pluripotential cells.

It is well known in the art that the genome of eukaryotic organisms is variably methylated through the addition of methyl ($-CH_3$) groups attached to cytosine residues in DNA to form 5'methylcytosine (5'-mC). Methylation is correlated with the control of gene expression. Typically genes that are hypomethylated tend to be highly expressed. Hypermethylation is correlated with reduced gene expression. It will be apparent to one skilled in the art that pluripotential cells will have a typical methylation pattern. This pattern may be analysed at a genomic level or at the level of a specific gene. Methods to analyse the extent of methylation are well known in the art and include, by example and not by way of limitation, restriction enzyme digestion of DNA with methylation sensitive restriction endonucleases followed by Southern blotting and probing with suitable gene probes (Umezawa *et al* 1997).

Alternatively or additionally said pluripotential characteristic includes the ability to induce tumours when introduced into an animal, ideally a rodent experimental model. More ideally still said animal is immunosupressed.

According to a second aspect of the invention there is provided a cell-line comprising cells according to the invention. Ideally, said cell-line are of human origin.

According to a third aspect of the invention there is provided a method for preparing a cytoplast, or part thereof, for use in the production of the cell or cell line of invention comprising;

i) providing at least one EC cell;
ii) separating at least part of the cytoplasm from the nucleus of said EC cell;
iii) isolating said cytoplasmic part; and, optionally
iv) storing said isolated cytoplasmic part prior to use.

In a preferred method of the invention said cytoplasmic part is a cytoplast.

It will be apparent to one skilled in the art that said cytoplast may be provided either as an aliquot isolated from at least one EC cell (eg an aliquot extracted from an intact EC cell via micromanipluation techniques) or alternatively, and preferably, said cytoplasmic part may be provided as an isolated cytoplast.

In a preferred method of the invention said cytoplast part is separated from said nucleus by exposure to a pharmacologically effective amount of at least one cytochalasin. Ideally cytochalasin B.

It is well known in the art that cytochalasin B is an example of a chemical effective at separating the nucleus of a cell from the cytoplasm to form a karyoplast and cytoplast respectively, (Methods in Enzymology Vol 151, p221-237 1987).

According to a fourth aspect of the invention there is provided a method for preparing a cell or cell-line in accordance with the invention comprising;

i) combining at least one EC cell with at least one somatic cell;

ii) removing from said combined cell, the EC cell nucleus;

iii) culturing said cell under conditions conducive to proliferation and expansion of said cell; and, optionally

iv) storing said cell culture under suitable conditions.

It will be apparent to one skilled in the art that methods of micromanipulation exist that facilitate the removal of nuclei from selected cells. It will be apparent that this method of the invention advantageously provides that ;

i) the factors produced by the EC cell are continually produced thereby maintaining a steady-state level of factors necessary to reprogramme the somatic cell nucleus; and

ii) the EC cell nucleus is removed from the combined cell prior to mitosis ensuring oncogenic genes are not transferred.

It will be apparent to those skilled in the art that the nature of the somatic cell selected is not critical to the operation of the invention although the cell-type will be selected so as to optimise or maximise success in terms of prodcution of a cell or cell-line of the invention.

According to a fifth aspect of the invention there is provided a method of combining at least part of the cytoplasm of an EC cell with a somatic cell comprising;

i) providing at least part of the cytoplasm of an EC cell;

ii) combining said cytoplasmic part with at least one somatic cell;

iii) growing said combined cell in culture; and, optionally

iv) storing said combined cell under suitable storage conditions.

In a preferred method of the invention said cytoplasmic part is provided as a cytoplast.

In yet a further preferred method of the invention said cytoplast is combined with said somatic cell via cytoplast/somatic cell fusion.

In the above described methods the EC cell and somatic cell are, ideally of human origin.

According to a sixth aspect of the invention there is provided a cell culture comprising at least one cell according to the invention.

According to a seventh aspect of the invention there is provided a method for inducing differentiation of at least one cell of the invention comprising:

i) providing a cell according to the invention;
ii) culturing said cell under conditions conducive to the differentiation of said cell into at least one tissue; and,optionally
iii) storage of said differentiated tissue prior to use under suitable storage conditions.

Ideally said culture conditions are selected so as to provide a tissue type, by example and not by way of limitation, that is, neuronal, muscle (eg smooth, striated, cardiac), bone, cartilage, liver, kidney, respiratory epithelium, haematopoietic cells, spleen, skin, stomach, intestine.

According to a eighth aspect of the invention there is provided at least one tissue type or organ comprising at least one cell according to the invention.

It will be apparent to one skilled in the art that differentiated tissue according to the invention may have extensive application with respect to transplantation therapy. For example, and not by way of limitation, replacement of damaged and/or diseased coronary and/or major arteries; replacement of damaged and/or diseased organs (eg as a result of kidney disease, (eg cirrohosis), diabetes, various autoimmune diseases); replacement of damaged neurones (eg Alzhiemers disease, Parkinsons disease, spinal injuries) or cancer. It will also be apparent to one skilled in the art that diseases such as AIDS may benefit from from tissues derived from the cells of the invention. The depletion of T-cells through virus induced cell death is the major contributory factor to the immuno-compromised state of AIDS suffers. The provision of a non-exhaustive supply of T-cells derived from a non-infected somatic cell from the patient has obvious benefits. Moreover, tissue rejection due to host cell immune responses are likely to be negligible since the somatic nucleus used in the cybrid would ideally be derived from the patient requiring the replacement tissue or organ.

According to an nineth aspect of the invention there is provided a therapeutic composition comprising at least one cell of the invention including a suitable excipient, diluant or carrier.

In a preferred embodiment of the invention said therapeutic composition is provided for use in tissue transplantation.

According to a tenth aspect of the invention there is provided a method to treat conditions or diseases requiring transplantation of tissue comprising;

i) providing at least one tissue type or organ according to the invention;
ii) surgically introducing said tissue type or organ to a patient to be treated; and

iii) treating said patient under conditions which are conducive to the acceptance of said transplanted tissue by said patient.

According to a eleventh aspect of the invention there is provided a kit comprising; at least one cell according to the invention; instructions with respect to the maintenance of said cell in culture; and, optionally, factors required to induce differentiation of said cell to at least one desired tissue type or organ.

An embodiment of the invention will now be described by example only and with reference to the following tables and figures wherein;

Table 1 represents a summary of the human EC cell lines and some of the characteristic features of said human EC cell-lines ;

Table 2 represents a summary of the murine EC cell lines used;

Figure 1 shows various human teratocarcinoma-derived cell lines. The characteristic embryonal carcinoma (EC) morphology of several human EC cell lines derived from testicular (a-e) and extragonadal (f-g) teratocarcinomas: (a) 1156QE, (b) TERA-1, (c) SuSa, (d) 833KE, (e) 1777NRpmet, (f) 1618K, (g) NCCIT. Bar = 50µm;

Figure 2 shows a flow cytofluorimetric analyses of surface antigen expression by the human EC cell line 2102Ep;

Figure 3 shows differentiation of several human EC cell lines caused by alteration in growth conditions: (a) 2102Ep cells placed at 10^5 per 75 cm^2 flask (low density); (b) spontaneous differentiation of a few cells in a culture of 1156QE; (c) a culture of SuSa cells passaged by trypsinisation; (d) a culture of

1777NRp differentiated cells derived from 1777NRpmet EC cells by repeated passage of low density (Bronson et al 1983a). Bar =50μm; and

Figure 4 shows differentiation of TERA-2 derived human EC cells: (a) NTERA-2 cl D1 human EC cells; (b) parental TERA-2 culture, showing mixed patches of EC and non-EC cells; (c) neurons and other differentiated cells arising in cultures of NTERA-2 cl D1 cells following induction with retinoic acid (Andrews 1984); (d) non-neural differentiated NTERA-2 cl D1 cells induced by exposure to hexamethylene bisacetamide.(Bar =50μm);

Figure 5 shows heterokaryons obtained by fusion of 2102Ep cl 4D3 human EC cells and the human T-cell leukaemia cell line, MOLT4. Several heterokaryons containing 2 and 3 nuclei can be seen in this field. (Bar = 50μm);

Figure 6 shows cytoplasts derived from NTERA-2 cl D1 human EC cells following enucleation with cytochalasin B. A remaining nucleated cell (top right) has been included in the field for comparison; and

Figure 7 shows PCR amplification of Oct4 mRNA from a human EC x somatic cell (thymocyte) heterokaryon.

Materials and Methods

Preparation of Mouse Thymocytes

The thymocytes were obtained by mincing a thymus removed from a 4-6 week old male mouse (Swiss strain) and suspending the released cells in 10 ml medium (DMEM) with 10% foetal calf serum (FCS). After standing for 2-3 minutes to allow large fragments of thymus to settle, the supernatant was removed and centrifuged at 1500 rpm for 5 min to pellet the suspended

thymocytes. The thymocytes were resuspended in fresh medium without FCS, and pelletted again by centrifugation; this was repeated a second time after which the cells were resuspended in fresh serum free medium and counted. Human EC cells were obtained by trypsinisation of confluent cultures as previously described (Andrews *et al.*, 1980; 1982). After washing two times in serum free DMEM, and counting, the human EC cells were mixed with the mouse thymocytes in a ratio of 1 EC cell to 10 thymocytes. The mixed cells were pelletted by centrifugation at 1500 rpm for 5 min.

Heterokaryon Fusion of Human EC cells and Mouse Thymocytes & Extraction of RNA

The cells were fused using polyethylene glycol (PEG) (Kennett, 1979). The pellet (in Experiment 1, 2 x 10^6 EC cells and 2 x 10^7 thymocytes; in Experiment 2, 3 x 10^6 EC cells and 3 x 10^7 thymocytes) was resuspended in 200 µl 50% (w/v) PEG 1500 in 75 mM HEPES, pH8.0 (Boehringer Mannheim) and incubated at 37° C for 1.5 min. Serum free medium, pre-warmed to 37° C, was then added gradually over 5 min. The cells were then pelletted by centrifugation at 1500 rpm for 5 min. and resuspended in 5 ml DMEM with 20% foetal calf serum. These cell were then plated into a T25 flask and placed in a humidified incubator (10% CO_2 in air) at 37°C for 2 days.

After 2 days, the non-attached cells were aspirated. The remaining attached cells were harvested by trypsinisation, and washed two times in DEPC-treated PBS to remove the serum. The pellet was then resuspended into Tri reagent (1 ml) to isolate RNA (Sigma-Aldrich Chemical Co., as described in Sigma Technical Bulletin MB-205). The isolated RNA was quantified by optical density measurements and the absence of contaminating DNA was determined

by PCR using β-actin and HPRT primers in separate samples (Wakeman *et al.*, 1998). If free of DNA, the RNA was then used for RT.PCR analysis of Oct4 expression.

PCR Amplification of Oct4 from Human EC x Mouse Thymocyte Heterokaryon

In one experiment (2102Ep with thymocytes), a control was prepared, consisting of cells treated as for fusion except that the incubation with PEG was omitted - thus it was anticipated that no 2102Ep x thymocyte heterokaryons would be formed. In another experiment RNA was isolated from thymocytes alone and also from a mouse EC line (PCC4 aza1, clone 3), to provide further negative and positive controls for mouse Oct4 expression. cDNA was then produced from the samples using reverse transcriptase (RT) (Wakeman *et al.*, 1998). PCR was then performed using oligonucleotide primers specific for human and mouse *Oct 4*, a marker of pluripotent cells under the standard PCR conditions described in Wakeman *et al.* (1998) with an annealing temperature of 61°C. These products were then subjected to electrophoresis and separated DNA fragments detected by ethidium bromide staining (Figure 7). Molecular size of the amplified fragments was determined by using a 1kb DNA step ladder.

PCR Primers for human and mouse Oct 4

Species	Annealing Temp (°C)	Sequence	Bp	GenBank Accession No. and primer location
Human Forward Reverse	61.4	5'-cgaccatctgccgctttgag-3' 3'-ccccctgtcccccattccta-5'	573	*X52437* 120-139 534-515
Mouse Forward Reverse	60.4	5'-gtccgcccgcatacgagttc-3' 3'-aggggccgcagcttacacat-3'	415	*Z11899* 361-380 937-918

These primers were designed using the PrimerSelect module of the Lasergene suite of programs (DNAStar Inc., USA). The mouse primers would not be expected to amplify human Oct4.

Enucleation of cells to yield 'cytoplasts' and 'karyoplasts' or 'mini-cells'.

One of the techniques that is employed in our method for producing Re-Programmed Embryonic Stem Cells (RPES cells) is the use of cytochalasin B to generate enucleated EC cells (EC cytoplasts) as the cytoplasm donor, and 'karyoplasts' (also called 'mini-cells') from the differentiated or committed cells as the nucleus donor. Cytochalasin B is well-known to induce cells to extrude their nuclei (Carter, 1967) and has been employed by numerous authors to induce enucleation of a wide range of cells of a variety of species including both mouse and human cells (Poste 1972; Prescott et al 1972; Goldman et al 1973; Wright and Hayflick 1973; Ege and Ringertz 1974a; Wigler and Weinstein 1975). Such enucleation results in a cell lacking a nucleus, but is otherwise intact and viable for a number of days (Goldman et al 1973); these enucleated cells have been called anucleate cells (Poste 1972) or cytoplasts (Veomett et al 1974). The nucleus that is extruded from the cell retains a thin rim of cytoplasm and is surrounded by a plasma membrane; these structures have been called 'karyoplasts' (Veomett et al 1974) or 'mini-cells' (Ege and Ringertz 1975). Enucleation of cells to yield both cytoplasts and karyoplasts may be achieved by well-established techniques in which cells growing attached to a plastic disc are inverted over a solution of cytochalasin B in a centrifuge tube and centrifuged; the cytoplasts remain attached to the plastic disc, while the karyoplasts are pelleted at the bottom of the centrifuge tube (Prescott et al 1972). Alternatively, cells in suspension may be centrifuged through a density gradient, typically composed of Ficoll, containing cytochalasin B (Wigler and Weinstein 1975). In this case, cytoplasts and karyoplasts are formed and may be recovered from different parts of the gradient after centrifugation.

Using the method described by Prescott et al 1972 NTERA-2 clD1 EC cells growing on a plastic disc were inverted over a solution of 7.5μg/ml

cytochalasin B, in phosphate buffered saline containing 10% fetal bovine serum, in a 50 ml centrifuge tube, and centrifuged for 30 minutes at 12,000 rpm and 37°C in a J2 Bectman Centrifuge using a JA20 rotor. Cytoplasts without nuclei remain attached to the disc. Occasional cells that have escaped enucleation also remain, please see Figure 6. After recovery by incubation overnight in medium without cytochalasin, the discs were fixed in methanol and stained with haematoxylin and cosin (Bar - 50μm).

<u>Methods for combining (fusing) the cytoplasm of one cell with the nucleus of another.</u>

The methods for creating hybrid cells by fusing two or more cells of different origins together are very well established and widely known. For a review of the commonly used methods based upon Sendai virus induced cell fusion, or cell fusion induced by polyethylene glycol (PEG), see Kennett (1979).

Briefly, mixtures of cells that it is desired to fuse are incubated with a fusogenic agent, such as Sendai virus or PEG, often with centrifugation or agitation to encourage clumping and close apposition of the cell membranes; variables such as time, temperature, cell concentration and fusogenic agent concentration are optimised for each cell combination. An example of cell fusion to produce heterokaryons is presented in Figure 6. Cell fusion was carried out using Polyethylene Glycol 1000, as described by Kennett 1979. Following fusion, the cells were seeded into tissue culture dishes, and incubated in fresh medium overnight. They were then fixed with methanol and stained with haematoxylin and cosin. Several heterokaryons containing 2 and 3 nuclei can be seen in this field. (Bar = 50μm).

These techniques have also been shown to allow fusion of cytoplasts, prepared by cytochalasin B induced enucleation, with whole cells or karyoplasts, also derived by cytochalasin B induced enucleation (Poste and Reeve 1971; Ege and Ringertz 1975; Ege et al 1973, 1974; Veomett et al 1974; Wright and Hayflick 1975; Shay 1977)).

Another technique that is now well established and widely used for inducing cell fusion, 'electrofusion', involves passing short electric pulses through mixtures of cells (Neil and Zimmermann 1993).

Production of RPES cells

The production of RPES cells requires several steps:

1. the selection of appropriate differentiated cells (the Nucleus Donor) and, if necessary, the isolation of their nuclei,
2. the selection of EC cells (the Cytoplasm Donor),
3. the fusion of the differentiated cell nuclei with the EC cells, and
4. the removal of the EC cell nucleus, either before or after fusion.

The production technique may, in some cases, be optimised by pre-treatment of the differentiated cells, or contemporaneous treatment of the differentiated cell/ EC cell fused products, with various agents such as, but not limited to, inhibitors of DNA methylation, to enhance the ability of the differentiated cell nucleus to be re-programmed. After the production of the RPES cells additional methods are required to propagate the cells, to characterise their properties and to induce them to differentiate into required somatic cell types.

Differentiated cells to be used as Nuclear Donors

A large range of somatic cells derived from any tissue or organ of an adult mammal or human, or from embryos or foetuses, or from extra-embryonic tissues such as the trophoblast or yolk sac may be used as a source of nuclei for reprogramming. Particular somatic cell types include but are not limited to thymocytes, peripheral blood lymphocytes, epidermal cells such as from the bucal cavity, cumulus cells, or other stem cells isolated from biopsies of various tissues, such as the bone marrow, the nervous system and the gut. The technique may also be applied to various established cell lines, such as those derived from various tumours including, for example, but not limited to lymphoblastoid cell lines. The selected somatic cells used for the re-programming procedure may be used directly upon isolation or they may be cultured for a short time before further manipulation. In some instances such somatic cells may be combined entirely with EC cells as described below, or nuclei or karyoplasts may first be isolated from them, for example using agents such as cytochalasin B, as discussed above, or by other methods. For example, nuclei may also be isolated using established micromanipulation procedures, or other established cell fractionation procedures.

Parental EC cells to be used as Cytoplasm Donors

A large number of EC cell lines have been isolated from human teratocarcinomas, which occur predominantly, but not exclusively, as testicular germ cell tumours. Examples of available human EC cell lines are shown in Table 1. Similarly, EC cell lines derived from teratocarcinomas of the laboratory mouse have also been derived and are readily available. Examples of available mouse EC cell lines are shown in Table 2. To date EC cell lines have not been described from any other species, but if they were derived in the future we would anticipate that they should behave in a manner similar to the existing human and mouse EC cells which resemble one another. Thus, newly isolated EC cells of other species, or from human or mouse

sources, should also be able to re-program differentiated cells to RPES cells as described in its proposal for human and mouse EC cells.

Human EC cells can be readily recognised by a combination of features that include their morphology (see Figure 1), their expression of the cell surface antigens SSEA3 (Shevinsky et al 1982, Andrews et al 1982, 1984b, 1996), SSEA4 (Kannagi et al 1983, Andrews et al 1984b, 1996), and TRA-1-60 and TRA-1-81 (Andrews et al 1984a, 1984b, 1996), and typically by low expression or absence of SSEA1 (Andrews et al 1980, 1982, 1984b, 1996) (see Figure 2). By contrast, mouse EC cells typically express SSEA1 (Solter and Knowles, 1978) but not SSEA3 (Shevinsky et al 1982), SSEA4 (Kannagi et al 1983) or TRA-1-60 or TRA-1-81 (Andrews et al 1984a). Human EC cells like mouse EC cells express high levels of alkaline phosphatase (ALP) (Bernstine et al 1973, Benham et al 1981); in the case of human EC cells most ALP activity is due to expression of the liver/bone/kidney isoform which can be detected as a cell surface antigen by monoclonal antibodies TRA-2-59 and TRA-2-54 (Andrews et al 1984c). In common with ES cells and primordial germ cells, EC cells also typically express the transcription factor Oct3/4 (Rosfjord and Rizzino 1994; Brehm et al 1998).

Fusion of parental differentiated cells and parental EC cells to yield RPES cells

Several methods may be used to combine the cytoplasm of an EC cell and the nucleus of a differentiated cell to yield an RPES containing the nuclear genome of the differentiated cell but not the EC cell.

A. Cells may be fused by use of chemical agents such as polyethylene glycol (PEG) or viruses such as Sendai virus, or by passing an electric current through a mixture of cells. As discussed above, these methods

are well known and may be readily applied. These methods may be used to fuse:

1. a differentiated cell with an EC cell, or
2. a karyoplast from a differentiated cell with an EC cell, or
3. a differentiated cell with one or more cytoplasts isolated from EC cells, or
4. a karyoplast from a differentiated cell with one or more cytoplasts isolated from EC cells.

In cases (1) and (2), the result will initially be a heterokaryon containing two nuclei, one from each parental cell. If this heterokaryon were allowed to divide the result would be a hybrid cell containing a single nucleus with a complete or partial genome from each parental cell. However, in our method of producing RPES cells, the EC nucleus is removed prior to cell division of the hybrid cell, so that the derivative dividing cell population retains only the genome of the parental differentiated cell.

In cases (3) and (4) the EC nucleus is removed from the EC cell before fusion, for example by enucleation with cytochalasin B as discussed above, so that the resulting product contains only the differentiated cell nucleus and cytoplasm from the EC cell parent. In any of these cases, the resulting RPES cells that continue to proliferate retain only the nuclear genome of the differentiated parental cell, which is now reprogrammed to express a new pattern of gene activity.

In cases (1) and (2) the EC cell nucleus is removed from the heterokaryon in one of several ways that include, but are not limited to, partial enucleation using drugs such as cytochalasin B, applied in the same manner as described above for enucleating EC cells and

generating cytoplasts for fusion. In the present case in which enucleation is carried out after fusion, some heterokaryons lose both nuclei, in which case they do not proliferate, some heterokaryons lose the differentiated cell nucleus, in which case they retain the parental EC nucleus and continue proliferating, some heterokaryons lose the EC cell nucleus, in which case they continue proliferating as RPES cells, and some heterokaryons retain both nuclei and eventually continue proliferating as hybrid cells. Several methods are used to select the RPES cells and to eliminate any of the cells retaining an EC cell genome or to eliminate any cells retaining a somatic nucleus that has failed to undergo re-programming. In one method, the proliferating cells are cloned by established techniques (e.g. by picking single cells with a micropipette - see Andrews et al 1982, 1984b), and individual clones are screened using genetic markers for those that retain an EC genome. The latter cells are discarded, whereas those that retain only a differentiated cell genome but not an EC cell derived genome, and express an RPES phenotype, are retained. Standard DNA genotyping techniques using well established DNA fingerprinting technology (Jeffreys et al 1985, 1988; Yan et al 1996) may be used to identify whether the nuclear genome of any proliferating cells is derived from either the EC cell or differentiated cell parent, or both.

In another method, before use as a fusion partner, the EC cell parent is genetically marked by insertion of a gene that will allow selection against any cell carrying that gene; for example, the EC cell can be stably transfected with a vector encoding the Herpes Simplex Virus-1 Tk gene (HSV1-Tk), such that any cells carrying that gene can be killed by culture in the presence of a number of drugs including acyclovir (9-[(2-hydroxyethoxy)methyl]guanine) or FIAU (1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodouracil) (Borrelli et al 1988; Hasty et al

1991), or gancyclovir (Rubinstein et al 1993; McCarrick and Andrews 1992). In this method, following partial enucleation, the remaining heterokaryons are cultured in medium containing this drug, and only those that have lost the EC cell nucleus survive. Other selectable genetic systems can also be similarly used. Persisting parental differentiated cells that have not been reprogrammed are removed by cloning the surviving cells, or by selecting RPES cells by virtue of their expression of specific surface antigen markers that include, but are not limited to, SSEA3, SSEA4, TRA-1-60 or TRA-1-81, as discussed above as characteristic markers of EC cells. These same markers have been shown to be expressed by ES cells derived directly from human embryos (Thomson et al 1998; Shamblott et al 1998) For the latter approach, fluorescence activated cell sorting (FACS), a widely used method for separating subsets of cells can be used (e.g. Andrews et al 1982, 1987; Ackerman et al 1994; Williams et al 1988).

In another method, the EC cell parent is incubated prior to fusion, with a drug that irreversibly inactivates its nucleus and prevents its replication, for example, topoisomerase inhibitors such as etoposide (Downes et al 1991; Fulka and Moor 1993). The resulting heterokaryon naturally eliminates this treated nucleus prior to cell division, so that the resulting dividing cell population only contains the genome derived from the parental differentiated cell. This approach may also be combined with the preceding ‘partial enucleation of heterokaryons’ approach to ensure complete loss of the EC genome.

In another method, after cell fusion to produce a heterokaryon, the EC cell nucleus is removed by micro-manipulation.

B. Rather than chemical, viral or electrically induced fusion, the nucleus of the differentiated cell is combined with an EC cell parent by micro-manipulation. In this method, the nucleus of the differentiated

cell is withdrawn using a micropipette inserted through the cell membrane. It is then injected either into an inoculated EC cell, or into an intact EC. In the later case the EC cell nucleus is then removed by a similar technique, or by one of the techniques described above, before nuclear fusion and cell division occurs.

Growth and selection of RPES cells

Following fusion to combine a differentiated cell and an EC cells, with prior or subsequent removal of the EC cell nucleus, it is necessary to provide appropriate conditions for the re-programming of the differentiated cell nucleus and for the subsequent proliferation of the resulting RPES cells.

Several methods are used to enhance the efficiency of reprogramming:

1. prior to fusion the differentiated cell and EC cell are synchronised with respect to position in the cell cycle, by use of reversible inhibitors that arrest the cell cycle at specific stages (e.g. nocodazole), or by the use of conditions such as low serum to arrest cells in G1, or by selection of cells at specific stages of the cell cycle by using vital DNA stains and flow microfluorimetry (Fluorescence Activated Cell Sorting) (Ashihara and Baserga 1979; Andrews et al 1987; Crissman 1995; Stein et al 1995).
2. the differentiated cell or the immediate fusion product is cultured in the presence of drugs that inhibit methylation or promote demethylation (e.g. 5-azacytidine) (e.g. Taylor and Jones 1979; Jones 1985; Keshet et al 1986), or alter the structure of chromatin, for example butyrate, spermine, trichostatin A or trapoxin which inhibit deacetylation and promote acetylation of histones, which plays a role in X chromosome inactivation, gene imprinting and

regulation of gene expression (Caldarera et al 1975; McKnight et al 1980; Stein et al 1997; Hu et al 1998; Wolffe and Pruss 1996;).

3. the period of time between production of heterokaryons and the removal of the EC cell nucleus is made as long as possible without permitting nuclear fusion. This period can be elongated by culturing the heterokaryons under conditions that reversibly inhibit progress through the cell cycle (e.g. thymidine block - Stein et al 1995), or by altering growth conditions, such as serum starvation or lowered temperature, that retard cell division but permit reprogramming to proceed.

4. any, or all combinations of these methods.

In all these experiments the cells are cultured in standard cell culture media that include but are not restricted to Dulbecco's modified Eagle's Medium (DME, high glucose formulation) or Ham's F12, supplemented in some cases with foetal bovine serum or with other additives (e.g. see Andrews et al 1980, 1982, 1984, 1994). Subsequent to fusion and re-programming, the growth of the resulting cells may be optimised culture on feeder layers of cells that include, but are not restricted to, irradiated or mitomycin C treated STO cells, or embryonic fibroblasts of various species, including humans (see Robertson 1987a; Thomson et al 1998). The cells may be cultured in the presence of various growth factors or other tissue culture additives, that include but are not restricted to LIF, FGF, SCF.

<u>**Differentiation of the RPES cells**</u>

In the best cases, the RPES cells acquire pluripotent properties that closely resemble those of embryonic stem cells, so that the RPES cells are able to differentiate and to initiate differentiation pathways that result in the formation of any cell type that may be found in the adult, embryo or in extra-embryonic

tissues, given appropriate conditions. The maintenance of an EC cell state can be monitored by assay of various markers that include the cell surface antigens SSEA3, SSEA4, TRA-1-60, TRA-1-81, by their expression of alkaline phosphatase and by expression of Oct3/4, as discussed above. The RPES cells typically retain their stem cell phenotype when cultured on appropriate feeder cells. However, they can initiate differentiation under a variety of circumstances.

Thus removal from feeder cells, or culture in suspension, followed by replating in the absence of feeder cells in appropriate tissue culture flasks results in differentiation of stem cells into a variety of cell types that include neurons, muscle of various sorts and haematopoietic cells (see descriptions in Robertson 1987a). Differentiation of pluripotent stem cells (e.g. see Figures 3 and 4) may also be initiated by altered conditions affecting cell density and aggregation (e.g. seeding at low cell densities or trypsinisation) or exposure to various agents that include but are not restricted to retinoic acid, and other retinoids, hexamethylene bisacetamide, and the bone morphogenetic proteins (see Robertson 1987a; Andrews 1984; Andrews et al 1982, 1990, 1994, 1996; Thomson et al 1998). The type of cells that arise depend upon the nature of the inducing agent, and the culture conditions including the presence or absence of specific growth factors or other molecules.

Discussion

Although pluripotent stem cell lines have been derived from early embryos (Robertson, 1987b; Thomson et al 1995, 1998), primordial germ cells (Matsui et al 1992; Shamblott et al 1998) and from germ cell tumours (reviewed, Andrews, 1998) of various species, including the laboratory mouse, rhesus monkeys and humans, and nuclei from differentiated somatic adult cells have

been re-programmed to yield embryonic stem cells by transplantation to enucleated oocytes (Campbell et al 1996; Wakayama et al 1998), there are no reports that pluripotent stem cells, resembling embryonic stem cells with the capacity to differentiate into a variety of functional somatic cell types, can be produced by the re-programming of differentiated or committed embryonic or adult somatic cells, or extra-embryonic cells, without the use of oocytes.

We now describe methods by which embryonal carcinoma (EC) cells, derived from teratocarcinomas, can be used to re-program various somatic, differentiated cells, or other embryonic or extra-embryonic cell types, to a state from which they can then be induced to differentiate into one or more functional differentiated cell types that are distinct from the parental cells. In the best cases, but not necessarily in all cases, the re-programmed cells produced by this technique, called 'Re-programmed Embryonic Stem Cells' (RPES cells), resemble embryonic stem cells derived directly from early embryos, and can be induced to differentiate into a broad range of functional, differentiated cell types that include, but are not limited to, neurons, muscle (including skeletal and cardiac muscle) and haematopoietic cells. These RPES cells are diploid with a normal karyotype, and isogenic with the differentiated parental cells from which they are derived. They may be used to generate differentiated cells for transplantation and use in cell and tissue replacement therapies.

In some cases, only partial reprogramming occurs with, for example, the activation of several genes that are not active in the parental differentiated nuclear donor cell. Such cells are also of use in a variety of these same circumstances.

An example of such a gene is Oct4. Oct4 has previously been reported to be characteristically expressed by undifferentiated EC and ES cells (Brehm *et al.*,

1998). Therefore, to test the ability of human EC cell cytoplasm to reprogram somatic cells, isolated mouse thymocytes were fused with human EC cells, (2102Ep, clone 4D3 (Andrews *et al.*, 1982) or TERA1 (Fogh and Trempe, 1975; Andrews *et al.*, 1980)), to produce heterokaryons which were tested after 2 days for activation of Oct4 expression from the thymocyte genome. Evidence for such activation would indicate, not only that human EC cells are capable of re-programming a somatic cell nucleus to an ES/EC cell like state, but also that the regulatory factors involved are capable of working between different mammalian species. Thus if human EC cells can reprogram a mouse somatic cell, we would anticipate not only that they would be able to reprogram a human somatic cell, but also that mouse EC cells would be able to reprogram human somatic cells as well. Similarly, given the resemblance of EC and ES cells, it would be expected that ES cells could reprogram somatic cells in the same way as EC cells.

In Experiment 1, as anticipated, an amplified band (573 bp), corresponding to human Oct4 expression was detected similarly in RNA preparations from the 2102Ep x thymocyte fusion in the presence of PEG, and in the mock fusion in the absence of PEG, consistent with its expression by 2102Ep human EC cells. However, a band corresponding to mouse Oct4 (415 bp) was only detected in the RNA preparation from the 2102Ep x thymocyte fusion in the presence of PEG, when heterokaryons were expected to be present. The corresponding absence of mouse Oct4 from the mock fusion indicates both the absence of Oct4 expression from mouse thymocytes in this experiment, and the requirement for formation of heterokaryons for its activation from the thymocyte genome by the 2102Ep cytoplasm. No products were seen in the 'water' control, indicating absence of contamination.

In a second experiment, in which 2102Ep and TERA1 human EC cells were fused with mouse thymocytes in the presence of PEG, mouse Oct4 was only

detected in the 2102Ep fusion, again confirming the ability of 2102Ep cells to reprogram mouse thymocytes with activation of Oct4 expression, but suggesting in this experiment that TERA1 cytoplasm did not achieve reprogramming. In both cases, human Oct4 was detected as expected, consistent with its expression by 2102Ep or TERA1 human EC cells. In further controls, no mouse Oct4 expression was detected in RNA prepared from isolated mouse thymocytes not used for fusion. However, a similar sized PCR band to that detected in the 2102Ep x thymocyte fusion samples, corresponding to mouse Oct4, was detected in mouse PCC4 EC cells as expected.

In our method, RPES cells are created by combining the nucleus from a differentiated or committed cell (the Nuclear donor), whether from adults or from embryos, with the cytoplasm from an EC cell (the Cytoplasm donor), from which the nucleus is removed. Several methods can be used to combine the nucleus from the differentiated cell and the cytoplasm from the EC cell; in some methods the EC cell nucleus is removed prior to combination of the cytoplasm with the donated nucleus, and in other methods the EC cell nucleus is removed after combination. If EC cells and differentiated cells from the same species are used, then the resulting RPES cells retain cytoplasmic genetic determinants (e.g. the mitochondrial genome) and a nuclear genome from the same species. By contrast, embryonic stem-like cells produced by transplantation of somatic cells into enucleated oocytes of other species will continue to harbour mitochondria of that other species. Especially for the production of human RPES cells and their differentiated derivatives for transplantation into a human host, the maintenance of a human nuclear and human cytoplasmic genome could be a distinct advantage. Further, RPES cells that are isogenic with the anticipated human host can be produced by this technique without resort to any embryo, so avoiding practical difficulties that may be associated, for example, with immune rejection upon transplantation to

the human host, and also obviating ethical difficulties inherent in the use of human embryos.

The method that we describe incorporates the techniques for maintaining and propagating the RPES cells produced, and the techniques for inducing them to differentiate into a range of differentiated, functional cell types.

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Table 1
Human EC Cell Cytoplasmic Donors (this list is illustrative but not comprehensive)

Cell Line	Phenotype in Culture [1,2]	Surface Antigen Expression [2,3]					Tumour Origin			Xenograft	References
		SSEA3	SSEA4	TRA-1-60	TRA-1-81	TRA-2-49 TRA-2-54 (L-ALP)[4]	Original Site	Biopsy Site	Histology		
1218E	EC	+	+	+	+	+	Testis	Primary	EC,S	EC	Andrews *et a l* 1980 Wang *et al* 1980,1981 Bronson *et al* 1984 Andrews *et al* 1996 Wenk *et al* 1994
833KE	EC	+	+	+	+	+	Testis	Primary	EC,T,C,S	EC	Bronson *et al* 1978, 1980, 1984 Andrews *et al* 1980, 1996 Wang *et al* 1980, 1981 Wenk *et al* 1994
TERA1	EC	+	+	+	+	+	Testis	Lung metastasis	EC,T		Fogh & Trempe 1975 Wang *et al* 1981 Bronson *et al* 1984 Andrews *et al* 1996 Wenk *et al* 1994
SuSa	EC	+	+	+	+	+	Testis		EC,T		Hogan *et al* 1977 Andrews *et al* 1996 Wenk *et al* 1994
NCC-IT	EC	+	+	+	+	+	Extragonodal	Primary	EC,T,Y	EC,T,C,Y	Teshima *et al* 1988 Andrews *et al* 1996 Wenk *et al* 1994
1618K	EC	+	+	+	+	+	Extragonodal		EL		Vogelzang *et al* 1983 Andrews *et al* 1996 Wenk *et al* 1994
1777N Rpmet	EC	+	+	+	+	+	Testis	Retroperitoneal Lymph node	EC		Bronson *et al* 1983, 1984 Andrews *et al* 1996 Wenk *et al* 1994
S2	? not EC	–	–	+/-	–	–	Testis	Primary	Seminoma	?EC features	Von Keitz 1994 Wenk *et al* 1994 Andrews *et al* 1996 Wenk *et al* 1994

Table 1
Human EC Cell Cytoplasmic Donors (this list is illustrative but not comprehensive)

Cell Line	Phenotype in Culture [1,2]	Surface Antigen Expression [1,3]					Tumour Origin			Xenograft	References
		SSEA3	SSEA4	TRA-1-60	TRA-1-81	TRA-2-49 TRA-2-54 (L-ALP)[4]	Original Site	Biopsy Site	Histology		
TERA2 including sublines such as NTERA-2 and clones	EC	+	+	+	+	+	Testis	Lung Metatasis	EC,T	EC,T	Fogh & Trempe 1975 Wang *et al* 1981 Andrews 1984 Andrews *et al* 1984b, 1990 Gönczi *et al* 1984 Thompson *et al* 1984 Fenderson *et al* 1987 Simeone *et al* 1990 Engstrom *et al* 1991 Miller & Dmitrovsky 1991 Andrews *et al* 1996 Wenk *et al* 1994
2102Ep	EC	+	+	+	+	+	Testis	Primary	EC,T,Y	EC	Andrews *et al* 1980, 1982 Wang *et al* 1980,1981 Bronson *et al* 1984 Andrews *et al* 1996 Wenk *et al* 1994
2102E Rpmet	EC	+	+	+	+	+	Testis	Retroperotameal lymphnode	EC,T		Wang *et al* 1980,1981 Bronson *et al* 1984 Andrews *et al* 1996 Wenk *et al* 1994
1156QE	EC	+	+	+	+	+	Testis	Primary	EC,C,S		Andrews *et al* 1980 Wang *et al* 1980,1981 Bronson *et al* 1984 Andrews *et al* 1996 Wenk *et al* 1994

Table 1
Human EC Cell Cytoplasmic Donors (this list is illustrative but not comprehensive)

Notes

1 The phenotype of cells in culture is based upon observations of morphology, growth patterns and antigen expression (see, Andrews *et al* 1996).

2 Culture conditions: Human EC cells can generally be maintained in Dulbecco's Modified Eagles medium (DMEM), high glucose formulation, supplemented with glutamine and 10% foetal bovine serum, but other media have also been used (e.g. RPMI for NCCIT) (see Andrews and Damjanov 1994). High cell densities (75 x 10^6 per 75 cm^2 tissue culture flask) are optimal for maintaining an EC phenotype (see Andrews *et al* 1982, 1984b, Andrews and Damjanov 1994). All the cell lines may be harvested for passage using 0.25% trypsin and 2mM EDTA, in Ca^{2+}/Mg^{2+} - free Dulbecco's phosphate buffered saline, but in some cases (TERA-2 and derivatives, and SuSa) clumping of cells is preferable for best maintenance of an EC phenotype. In the latter cases, cells are harvested for passage by scraping, for example with glass beads, rather than by use of trypsin (see Andrews *et al* 1984b, Andrews and Damjanov 1994).

3 Surface antigen expression: SSEA-3, SSEA-4, TRA-1-60 and TRA-1-81 expression is characteristic of human EC cell lines but the level of expression of these antigens is variable and appears to reflect their state of differentiation (e.g. see Andrews *et al* 1996, Andrews *et al* 1982, Kannagi *et al* 1983, Andrews *et al* 1984a, Shevinsky *et al* 1982.

4 High levels of the liver/bone/kidney isoforms of alkaline phosphatase (L-ALP) detected as a cell surface antigen by monoclonal antibodies TRA-2-49 and TRA-2-54 are also characteristic of human EC cells (Benham *et al* 1981, Andrews *et al* 1984c, Andrews *et al* 1996).

5 Differentiation and loss of EC phenotype: Many human EC cells undergo morphological changes and change in surface antigen expression, notably the induction of SSEA-1 and down regulation of SSEA-3, when cultured at low cell densities and well dispersed (Andrews *et al* 1982, 1984b). These changes appear to represent a limited capacity for differentiation. Other lines differentiate extensively if exposed to agents such as retinoic acid (e.g. NTERA-2, Andrews 1984; NCCIT, Teshima *et al* 1988), hexamethylene bisacetamide (e.g. NTERA-2, Andrews *et al* 1986, 1990), and the bone morphogenetic proteins (e.g. NTERA-2, Andrews *et al* 1994). Differentiation in the latter cases is marked by loss of the characteristic EC marker antigens, appearance of new antigens (e.g. see, Fenderson *et al* 1987), activation of new genes (e.g. *Hox* genes, Mavilo *et al* 1988; *Mal*, Wakeman *et al* 1997; *Wnt-13*, Wakeman *et al* 1998).

Table 2
Mouse EC Cell Cytoplasmic Donors (this list is illustrative but not comprehensive)

Cell Line	Surface Antigen Expression SSEA-1[1]	Reference
F9	+	Bernstine *et al* 1993, Solter and Knowles 1978
PCC4	+	Jakob *et al* 1973, Solter and Knowles 1978
PCC3 (ND1)	+	Jakob *et al* 1973, Solter and Knowles 1978
MH-15	+	Solter and Knowles 1978
FA-25	+	Solter and Knowles 1978

[1] Solter and Knowles 1978